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Nitric oxide triggered defense network in wheat: Augmenting tolerance and grain-quality related traits under heat-induced oxidative damage

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ABSTRACT

Heat stress (HS) drastically reduces the yield and quality of wheat grains. High temperature during critical stages (pollination and grain-filling) causes improper fertilization and formation of defragmented granules and shriveled seeds. Hormones and signaling molecules modulates the tolerance potential of the plants under stress. Nitric oxide (NO) is an important signaling molecule involved in triggering diverse physiological and biochemical processes under adverse conditions. Here, we studied the effect of sodium nitroprusside (SNP) (150 μM set based on pilot experiment) on heat stress-tolerance and grain quality related traits of two contrasting wheat cultivars Raj3765 as thermotolerant and HD2932 as thermosusceptible under differential HS (T_1 - 30 °C, 1 h; T_2 - 38 °C, 1 h), as compared to control (22 ± 2 °C) at different stages of growth. The expression of many important stress-associated genes (previously identified through Transcriptome sequencing) was observed upregulated in response to NO and HS; small *HSP17* showed maximum fold increase in Raj3765. Similarly, the networks of antioxidant enzymes (SOD, CAT, and GPX) were observed triggered in response to NO, HS and NO + HS treatment in Raj3765 than HD2932. The accumulation of proline and free amino acid in cytoplasm were also reported higher under NO and HS treatment respectively in Raj3765, as compared to HD2932. Grain quality related traits like carbohydrate (starch) and proteins (gliadin) were observed higher in response to NO under HS in Raj3765. Heat stress was observed to increase the activities of α/β amylases in developing grains involved in degrading the starch quality. NO was observed to decrease the amylolytic activity in both the cultivars; very low amylolytic activity was observed in thermotolerant, as compared to thermosusceptible cultivars. Exogenous application of NO (150 μM) at different stages can be used as inexpensive technology for mitigating the problem of terminal HS in wheat – a farmer friendly approach for maintaining the quality of grains under present threat of global climate change.

1. Introduction

Wheat is one of the most important cereal grain crops providing major share of carbohydrate and energy in the diets. Wheat is highly sensitive to heat stress and even slight fluctuation in temperature during critical stages decreases the quality and quantity of the grains (Goswami et al., 2015). Heat stress causes leakage in the cell membrane, denaturation/ aggregation of important enzymes, and imbalance in the redox potential of the cells. It also affects the pollination and fertilization, as evident from the drying of the stigmatic surface, sterility of the pollen, improper growth of the pollen tube and air-filled cavity in the stigma of wheat (Kumar et al., 2014). The photosynthetic

Abbreviations: SAGs, stress-associated genes; HS, heat stress; NO, nitric oxide; DHS, differential heat stress; qRT-PCR, quantitative real-time PCR

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rate decreases abruptly with every 1 °C increase in the temperature. The source to sink ratio is disrupted and the whole starch biosynthesis pathway can hampered (Kumar et al., 2013; Mostofa et al., 2014). The alteration in the activities of enzymes associated with starch biosynthesis pathway causes improper, defragmented and small starch granule synthesis with lots of empty pockets which decreases the weight of seeds and total yield (Kumar et al., 2016). Even the quality of the grains in terms of composition of total carbohydrate, lipids, proteins and minerals are compromised under HS. Exogenous application of chemicals such as phytohormones, osmoprotectants, etc. are known to enhance the HS tolerance to plants through modulating the expression and activities of stress-associated genes/ proteins (Chan and Shi, 2015; Kumar et al., 2014).

Nitric oxide (NO) is diffusible gaseous free radical having diverse functions in plant physiological process (Del Rio et al., 2004). NO is lipid and water soluble phytohormone involved in signaling processes in plant and animal system. The emission of NO has been reported several years ago in soybean plants (Klepper, 1979; Domingos et al., 2015). Previously, in vivo and in vitro NR-dependent NO production was found in sunflower and maize crops (Rockel et al., 2002). NO is involved in various plant responses such as photosynthesis, oxidative defense, osmolyte accumulation, gene expression, and protein modifications under heat stress (Parankusam et al., 2017). NO along with other phytohormones such as abscisic acid (ABA) and jasmonates was reported to have functions in mitigating or activating the proteins associated with stress tolerance in diverse plant species (Del Rio et al., 2004). NO also function as an antioxidant by directly scavenging the superoxide radicals to form peroxynitrite (Radi et al., 1991), which is less toxic than peroxides and causes less cellular damage. NO also regulate the expression of defense related genes by acting as a signaling molecule (Lamattina et al., 2003).

Plant under abiotic stress undergoes oxidative and nitrosative stress. NO helps in activating the defense network associated with scavenging of ROS under stress. Interaction between NO and active oxygen play an important role as an antioxidant in different stress. Exogenous application of NO is helpful in mitigating the effect of HS-induced oxidative damage in plant. NO regulates the toxicity of ROS by modulating the ROS production and degradation (Del Rio et al., 2004). It helps in regulating the transcriptional changes involved in different process like signal transduction, basic metabolism, etc. (Palmieri et al., 2008).

NO has been reported to mitigate the effect of HS damage in rice seedling and increased the survival rate of wheat and maize crops (Uchida et al., 2002). NO triggers the activity of antioxidant defense network regulating the level of ROS in plants during HS (Neill et al., 2002; Song et al., 2006). The homeostasis of NO/RNS has been reported to play very important role in heat acclimation under HS by neutralizing the excessive oxidative and nitrosative injury in plants. Certain downstream signaling pathways, employing calcium ion (Ca^{2+}), cyclic guanosine monophosphate (cGMP), and cyclic adenosine diphosphoribose (cADPR), were proposed to be involved in NO-mediated molecular events in plant cells (Besson-Bard et al., 2008). NO also modulate the biological responses by direct modification of proteins, reacting with cysteine residues (*S*-nitrosylation), tyrosine residues (nitration), or iron and zinc in metalloproteins (metal nitrosylation) (Besson-Bard et al., 2008).

The present investigation is undertaken to understand the role of exogenous application of NO on HS-tolerance and grain-quality related traits of wheat under differential HS.

2. Material and methods

2.1. Sample and stress treatments

Seeds of two most popular and contrasting wheat cultivars *i.e.* Raj3765 and HD2932 were collected from Division of Genetics, Indian Agricultural Research Institute (IARI) and were sown in National Phytotron Facility under regulated conditions (22 ± 3 °C, 16 h light/ 8 h dark cycle). Pots filled with equal quantity of FYM and fine sands were used for the sowing of seeds. The germinated seedlings having 2–3 true leaves were thinned to 10 plants per pot. We used 48 pots for each cultivar divided into group of four based on the treatments of NO and HS - Group I – control under ambient conditions; Group II – Plants were exposed to NO and differential HS treatments during pre-anthesis stage; Group III – Plants were exposed to NO and differential HS treatments during milky-ripe stage; Group IV—NO and differential HS treatments were given during mealy-ripe stage. The treatments were given continuously for three days and samples were collected after 5 days of treatment for effective analysis. The flow chart for the HS treatments has been depicted in the supplementary figure (Fig. S1). We used sodium nitroprusside (150 μM) as source of NO based on the recommendation of Garcia-Mata and Lamattina, (2001). Pots were exposed to differential HS treatments (C- 22 ± 3 °C, T_1 – 30 °C for 1 h, T_2 – 38 °C for 1 h) inside microprocessor regulated chambers where temperature would increase/decrease in a pattern of 1 °C for every 10 min keeping appropriate relative humidity (75–80%) and other parameters constant. Samples were collected in three biological replicates at pollination, milky-ripe and mealy-ripe stages of growth and development based on the Feekes scale (Miller, 1999) and stored in -80 °C Ultra deep freezer for further downstream application. Because of 12 days difference between milky and mealy-ripe sub-stages of grain-filling, we have mainly focused our analysis on pollination and mealy-ripe stages in order to get the significant differences under NO and differential HS treatments. The harvested seeds were used for the analysis of traits associated with the quality of grains.

2.2. Infra-red gas analyzer analysis

The photosynthetic related parameters were estimated using Infra-red Gas Analyzer instrument (LI-COR, Lincoln, Nebraska, USA) (Kumar et al., 2017). The reading was taken during forenoon between 11.00 a.m. to 12:00 pm uniformly in all the biological replicates.

2.3. RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was isolated from the collected wheat leaves samples using Trizol reagents (Invitrogen, UK). The quality of the isolated total RNA was checked using Bioanalyzer (Agilent). RNA integrity was verified on 1.2% agarose gels. cDNA was synthesized using Revert Aid™ H minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The synthesized cDNA was quantified and further diluted to 100 ng/μL for qRT-PCR reactions. From the list of DETs identified through de novo transcriptomic sequencing of contrasting wheat cvs. under control and HS-treated conditions in our lab (BioProject acc. no. PRJNA171754), ten upregulated transcripts (ATP synthase, PS-II, POX membrane, Serine threonine kinase (STK), Zinc Finger CCH Domain, CDPK5, HSFA2b, UDP Glucosyl transferase, HSP70 and Glucan Synthase-like) and ten downregulated transcripts (Zinc Finger Protein Var3, Dnaj homolog, Myb Family TF, TF Myb44, Sucrose synthase2, sucrose phosphate synthase, sucrose transporter protien5, Delta Gliadin1, Alpha amylase inhibitor, alpha glucan water dikinase) were selected for the expression analysis (Table 1). Primers for qRT-PCR were designed using the Gene Fischer designing software (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/). We used three biological and three technical replicates for the expression analysis of stress associated genes (SAGs) (Kumar et al., 2016). The primer specificity and formation of primer-dimers were monitored by dissociation curve analysis and 3% agarose gel electrophoresis. β-actin gene (accession no. AF282634) was used as an endogenous control for normalizing the data. Relative fold expression was calculated using the Pfaffl method (Pfaffl, 2001).

Table 1
List of primers specific to upregulated and downregulated stress-associated genes used for the quantitative Real-Time PCR.

Oligo ID	Primer Sequence (5'-3')	Tm (°C)
	Upregulated	
Glucan synthase-like 2-f	TTCCTGTTCAACCCTTCTGG	60.0
Glucan synthase-like 2-r	CCATGACTCCCAGCTCTTGT	60.0
UDP-glycosyltransferase-f	CACGTTCGGCAGCTTACTT	59.0
UDP-glycosyltransferase-r	TCCGTCGGTTGAGTACGAAT	60.5
HSF A2b-f	ACGACATGGATGTGCTGTCT	59.1
HSF A2b-r	AGAAGTTGTGCCACCCGTAG	60.1
CDPK5-f	CCGTCACACAAGAGCAAGAA	60.0
CDPK5-r	TTGTGTGCTTGGAATGGAAA	60.0
Zinc Finger CCH domain-f	GAAGCAGCCTGTGATGAACA	60.5
Zinc Finger CCH domain-r	TACTTGAAGGCGGACCAGAT	60.2
Serine/threonine kinase-f	TCTGTGACCTCTCGAACACG	60.2
Serine/threonine kinase-r	GATTTCCCGAGGAAGTCTGA	59.5
Zm-HSP70-f	GAGCTGTGCAAGAGCATCAA	60.2
Zm-HSP70-r	CAGCTGTCTCCAGACCAAGAG	60.1
POX memb-f	CACTTCCTCTCCCTGGAACA	60.1
POX memb-r	CCAAAACATCCAAGCAGACA	59.8
PSII-f	GAGCAGGCTCAATGGACAAT	60.2
PSII-r	ACGGCGTACAAAAAGCTCAC	60.3
ATP synthase-f	CGCCTCTGCTCTCGATAAAA	60.0
ATP synthase-r	TTCCACTGGGAGAGGATTTG	60.0
HSF A6e-f	CGCAGAACATTCAGGAGCTT	60.0
HSF A6e-r	GGTCTCCTTCCCTCCAACTC	60.0
TaHD97-f	CCAACTTCTCCAGCTTCGTC	60.0
TaHD97-r	TGCTGCTGATGTCTTCATCC	59.9
HSP90-f	TGAGTATGGGTGGACTGCCAACAT	62.7
HSP90-r	TCTCGAAGAGCAGCATCACAAGGT	62.7
HSP70-f	CTTCGTCCAGGAGTTCAAGC	63.9
HSP70-r	GTCGATCTCGATGGTGGTTT	63.9
HSP26-f	ATCGACGTGAAGGTCCAGTGATGA	62.7
HSP26-r	AGAAAGGATCGGAGAAGAACGGCA	62.7
HSP17-f	AGTGGGTAGCGAGTTTCGTGTGAT	62.7
HSP17-r	CAAACAACCACCAGTAGCCACGAA	62.7
SOD-f	TCCTTTGACTGGCCCTAATG	63.8
SOD-r	CTTCCACCAGCATTTCCAGT	64.0
CAT-f	CAAGAGCGATTCATCAACAGAT	63.0
CAT-r	AGACCAGTAGGAGAGCCAGATG	63.6
APX-f	GCTCCCTACTGATAAGGCATTG	63.6
APX-r	TTTGTGTGATTCAGCGTAGTCC	63.9
Rca-f	CAGTCATTCATCACCGATCA	57.9
Rca-r	CAAAACATGCCCAGCAAT	58.0
MAPK-f	CTCCTGAATTGCTGCTTTGC	58.1
MAPK-r	GGAGTGTAGGGAAGGGTCTT	58.4
Actin-f	GCGGTCGAACAACTGGTATT	63.7
Actin-r	GGTCCAAACGAAGGATAGCA	63.8
	Downregulated	
TF MYB44-f	CCCAGAACTGGAACCTCATC	59.5
TF MYB44-r	TCAGCTTCTCCTCCTCTTCG	59.8
SPS 5-f	GCGCAACATCCACCTCTACT	60.2
SPS 5-r	GGAGCGATAGGTCCTGGAAT	60.4
STP 5-f	TAACCTTCTCCGTCGTGGTC	60.1
STP 5-f	TCCTCCAGGAATGACTCCAT	59.4
Alpha-GWD-f	GTGATGCTCCTGCATCTCAA	59.9
Alpha-GWD-r	TCCTTCCTTGCTTCCTCAAA	59.9
Alpha-amylase inhibitor-f	ATGGTGCAAGGAGCCATAAC	59.9
Alpha-amylase inhibitor-r	GCTGGCAGCAATCTCTTAGG	60.1
Zinc finger protein-f	ATGTCGAGGTTGTGGTGTCT	57.9
Zinc finger protein-r	ATTGCATTGTCCTCTTCACC	57.5
Delta gliadin 1-f	AAGACCTCGCAACAGAACAG	58
Delta gliadin 1-r	AATTGATGTTGAGGCTGCAT	58.1
Sucrose synthase 2-f	ACACCCCTTACTCGGAGTTC	58.1
Sucrose synthase 2-r	ATTGAACACCATTGGGATTG	58.1
Myb TF-f	ACCTGTTGAAAAGCTGAACG	58
Myb TF-r	GCTGGCAGTTCATTGCTTAT	58
DnaJ-f	TGAATGTTGGAGGTGGAGTT	57.9
DnaJ-r	TACCATGAGCTGAAGTGCAA	58

*HSF, Heat responsive transcription factor; CDPK, Calcium dependent protein kinase; HSP, Heat shock protein; POX, Peroxidase; PS-II, Photosystem II; ATP Syn, ATP synthase; SOD, Superoxide dismutase; CAT, Catalase; APX, Ascorbate peroxidase; Rca, RuBisCo activase; MAPK, Mitogen-activated protein kinase; SPS, Sucrose phosphate synthase; STP, Sucrose transporter protein; Alpha-GWD, Alpha-glucan water dikinase; Myb, Myb family transcription factor APL; DnaJ, DnaJ homolog subfamily C member 7.

2.4. Activity assay of antioxidant enzymes

2.4.1. Superoxide dismutase assay

Leaves (1 mg) were grounded in 6 mL of ice cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium EDTA and 1% (w/v) polyvinyl–polypyrrolidone (PVP). The homogenates were centrifuged at 10,000 × g (4 °C) for 10 min. The tissue extracts were either stored at −80 °C or immediately used for subsequent analyses of superoxide dismutase and catalase. For the quantification of soluble protein content, coomassie blue dye binding assay was used (Bradford, 1976). Bovine serum albumin (BSA) was used for the preparation of the standard curve. Superoxide dismutase activity was determined by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) in the presence of riboflavin in light (Giannopolitis and Ries, 1977). One unit of enzyme activity was determined as the amount of the enzyme needed for the inhibition of 50% NBT reduction rate by monitoring absorbance at 560 nm with spectrophotometer.

2.4.2. Catalase activity (CAT) assay

Activities of catalase enzyme were measured as described by Maehly and Chance, (1954). Fresh leaf material (1 *g*) was crushed in 5 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 10,000 *g* (4 °C) for 10 min. The supernatant were used for the quantification of soluble protein content by using Bradford method (Bradford, 1976). For assaying CAT activity, the decomposition of H_2O_2 was followed by decline in the absorbance at 240 nm. CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient - 39.4 $mmol^{-1}$ cm^{-1}) at 240 nm over a 3 min interval.

2.4.3. Guaiacol peroxidase (GPX) activity assay

Activity of guaiacol-peroxidase (GPX) was analysed following the method of Everse et al. (1994) with slight modification. Fresh leaf material (1 *g*) was crushed in 5 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 10,000 *g* (4 °C) for 10 min. The supernatant isolated were used for the quantification of soluble protein content by using Bradford method (Bradford, 1976). The oxidation of guaiacol into tetraguaiacol was estimated by measuring absorbance at 470 nm against the reagent blank. The activity of GPX was calculated in unit of μmol/min/mg protein using the extinction coefficient of 26.6 mM^{-1} cm^{-1}.

2.5. Estimation of total antioxidant potential

The FRAP (Ferric reducing antioxidant power assay) procedure described by Benzie and Strain, (1999) was followed for the total antioxidant activity assay. This method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants. The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to 1 mmol L^{-1} $FeSO_4$.

2.6. Estimation of proline accumulation

Proline contents were determined according to the protocol described by Bates et al., (1973) with slight modification. Leaf tissue (0.3 g) were grounded in 10 mL of 3% sulphosalicylic acid and then centrifuged at 10,000 × g for 10 min. The reaction mixture was prepared and later the absorbance value of toluene was measured at 520 nm with spectrophotometer as described previously (Kumar et al., 2015). The concentration of proline was calculated using a standard curve.

2.7. Free amino acid estimation

The free amino acid was estimated following the protocol of Sircelj et al., (2005). The leaf sample (0.2 gm) was crushed in liquid nitrogen and further 2 mL of 80% ethanol was added. The extract was centrifuged at 8000 g for 15 min and supernatant was collected. Reaction mixture was prepared by adding 100 µL of enzyme extract, 1 mL Ninhydrin reagent and 900 µL of water. The reaction mixture was incubated at 100 °C for 20 min and further OD was taken at 570 nm. The blank set was prepared by adding 0.1 mL of 80% ethanol in place of sample extract. The standard curve was prepared by using leucine as a standard amino acid and the free amino acid was expressed in mg of amino acid per g of tissue.

2.8. In vivo gliadin protein accumulation pattern in developing grains of wheat

The collected developing grains (1 g) from wheat cultivars Raj3765 and HD2932 (of different treatments) were crushed into fine powder using the liquid Nitrogen. The fine powder was serially diluted in 70% ethanol and fractionated; the alcohol soluble fraction was separated and concentrated using ultra Amicon column. The fractionated and concentrated samples were used for the SDS-PAGE as described by Laemmli, (1970). Concentration of protein was estimated by Bradford method (Bradford, 1976). 20 µg of protein samples were separated electrophoretically on SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) microporous membranes using the semi-dry iBlotter (Invitrogen, UK). We used primary antibody (polyclonal anti-gliadin synthesized in rabbit; Sigma) and secondary antibody (peroxidase-conjugated goat anti-rabbit IgG) in dilution of 1: 4000 for 1 h at RT. The membrane was developed by adding 10 mL of 0.05% 3, 3′diaminobenzidine (DAB) in TBS and 15 µL of H_2O_2 (30%). The dried membrane was used for the photography using Gel Doc Easy (Bio Rad, UK) as described previously (Kumar et al., 2017).

2.9. In-gel assay of amylases in developing wheat grains under NO and HS

The developing endospermic tissue of 100 mg (from each treatment) was ground in a pestle and mortar. Alcohol soluble protein fractions were extracted by washing the powdered sample in 50% (v/v) 1-propanol, 50 mM Tris-HCl (pH 7.5) and 1% DTT at 60 °C for 30 min. The supernatant was removed by centrifugation at 9500 g for 5 min. The protein estimation was carried out using the Bradford method (Bradford, 1976).

Further, 25 µg of alcohol soluble protein fraction (from each treatment) was loaded on to 10% native PAGE gel as described previously (Kumar et al., 2015). The gel after the electrophoresis was immersed in solution of 1% starch dissolved in Tris-Cl buffer (pH 7.3) followed by 2 min washing in distilled water. The gel was further stained in 10 mM iodine along with 14 mM potassium iodide for 5 min. Excess iodine was removed with cold distilled water and the gel was soaked in 1% acetic acid before capturing the image using Gel Doc Easy (Bio Rad, USA).

2.10. In-vitro assay of total amylolytic, α-amylases and β-amylase activities in wheat grains under NO and HS

Developing wheat endospermic tissue (0.5 g) was taken in pre-chilled pestle and mortar and homogenized thoroughly with 2 mL ice cold calcium-acetate buffer containing 10 mM each of sodium acetate and calcium chloride (pH 6.0) as per the modified protocol of Briggs, (1967). The extract was centrifuged at 10,000 x g for 5 min and the supernatant was separated in fresh tube. The reaction mixture was prepared in 1 mL of calcium acetate buffer (pH 4.8) and 1 mL of substrate starch solution (1% w/v in reaction buffer). Further, 100 µL of enzyme extract was added and the reaction was incubated at 24 °C for 10 min. The enzyme reaction was stopped with 1 mL of color reagent (60 mg each of KI and I_2 in 100 mL of 0.05 N HCl. The color change over time was read at 620 nm against blank. Total amylase activity was calculated using the standard curve of starch and expressed as µmol/min/mg protein. For the estimation of α-amylase activity, the extract prepared was pre-heated at 70 °C for 2 min in order to inactivate the β-amylase and other hydrolytic and proteolytic enzymes.

β-amylase activity was measured followed the protocol of Bernfeld, (1955). Reaction mixture was prepared by taking 0.5 mL of suitably diluted enzyme and 1% starch prepared in 50 mM sodium acetate buffer (pH 5.0). This was incubated at 30 °C for 3 min, and reaction was stopped by addition of 1 mL of 3, 5-dinitrosalicylic acid. Reaction mixture was then placed in boiling water bath for 5 min and was allowed to cool down to room temperature, followed by addition of 10 mL of distilled water. Absorbance was recorded at 540 nm. One unit of *β*-amylase is defined as the amount required for release of 1 µM of *β*-maltose per min at 30 °C and pH 5.0 under the specified condition.

2.11. Statistical analysis

Three biological and technical replicates were used for molecular and biochemical analysis at different stages of growth. The analyses of variance of different parameters were calculated using the one-way ANOVA for statistically significant differences ($P \leq 0.05$).

3. Results and discussion

With the burgeoning population and decrease in yield and quality of produce of agriculturally important crops, there is a need to understand and develop the cost-effective technologies to modulate the tolerance level of the plants against various abiotic and biotic stresses without compromising the quality of yield. Terminal HS is one of the major problems in wheat growth and development. The loss is very apparent from last few years.

3.1. Effect of NO on physiological parameters associated with photosynthesis under differential HS

In case of Raj3765, we observed decrease in the P_n with increase in temperature; minimum P_n was observed in response to T_2. NO was observed to modulate the P_n of Raj3765 and in turn mitigate the harmful effect of HS on P_n. Similar pattern was observed in case of conductance of Raj3765 under NO and HS treatments (Fig. 1a). We observed non-significant variations in the transpiration rate of Raj3765 in response to treatment; reason being the very low value of transpiration rate. The Infra-Red Gas Analyzer based physiological parameters (P_n, conductance and transpiration rate) was observed very low in HD2932, as compared to Raj3765 under NO and HS treatments (Fig. 1b). The involvement of NO in reproductive processes, development and other physiological responses like photosynthesis and stomatal closure has been previously reported (Wilson et al., 2008).

The plants exposed to NO and differential HS (T_1 and T_2) during mealy-ripe sub-stage of grain-filling was analyzed for photosynthetic related physiological parameters. We observed increased photosynthetic rate in Raj3765 during mealy-ripe, whereas decreased in response to differential HS (T_1 and T_2). The P_n value was observed minimum under T_2 treatment. The pattern of conductance was similar to P_n in Raj3765 under NO and differential HS (T_1 and T_2) treatments. Non-significant variations in the transpiration rate was observed in response to NO and differential HS (T_1 and T_2) during mealy-ripe stage. IRGA based analysis in HD2932 during mealy-ripe stage showed increase in the P_n, whereas decrease in the conductance and transpiration rate in response to NO treatment. Differential HS (T_1 and T_2) was observed to decrease all three parameters, as estimated using IRGA during mealy-ripe stage in HD2932. Combined treatment of NO and differential HS (T_1 and T_2) during mealy-ripe stage was observed to mitigate the effect of HS on photosynthetic related parameters. The P_n,

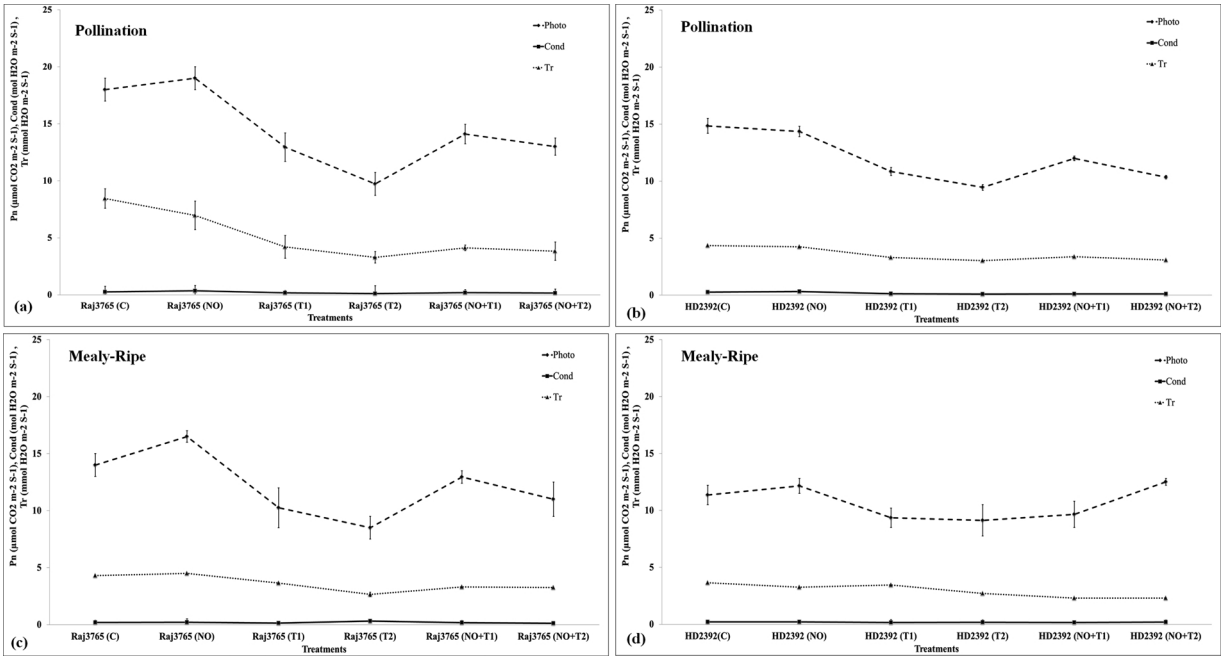

Fig. 1. Effect of exogenous application of NO on various physiological parameters associated with photosynthesis in contrasting wheat cultivars under differential HS, (a & b) Photosynthetic rate, conductance and transpiration rate of wheat cultivars Raj3765 and HD2932 during pollination stage in response to HS (T_1 and T_2), (c & d) Photosynthetic rate, conductance and transpiration rate of wheat cultivars Raj3765 and HD2932 during mealy-ripe sub-stage of grain-filling under T_1 and T_2 treatments; C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h; IRGA was used for the estimation of various photosynthetic related parameters; Three biological replicates were used for the experiment.

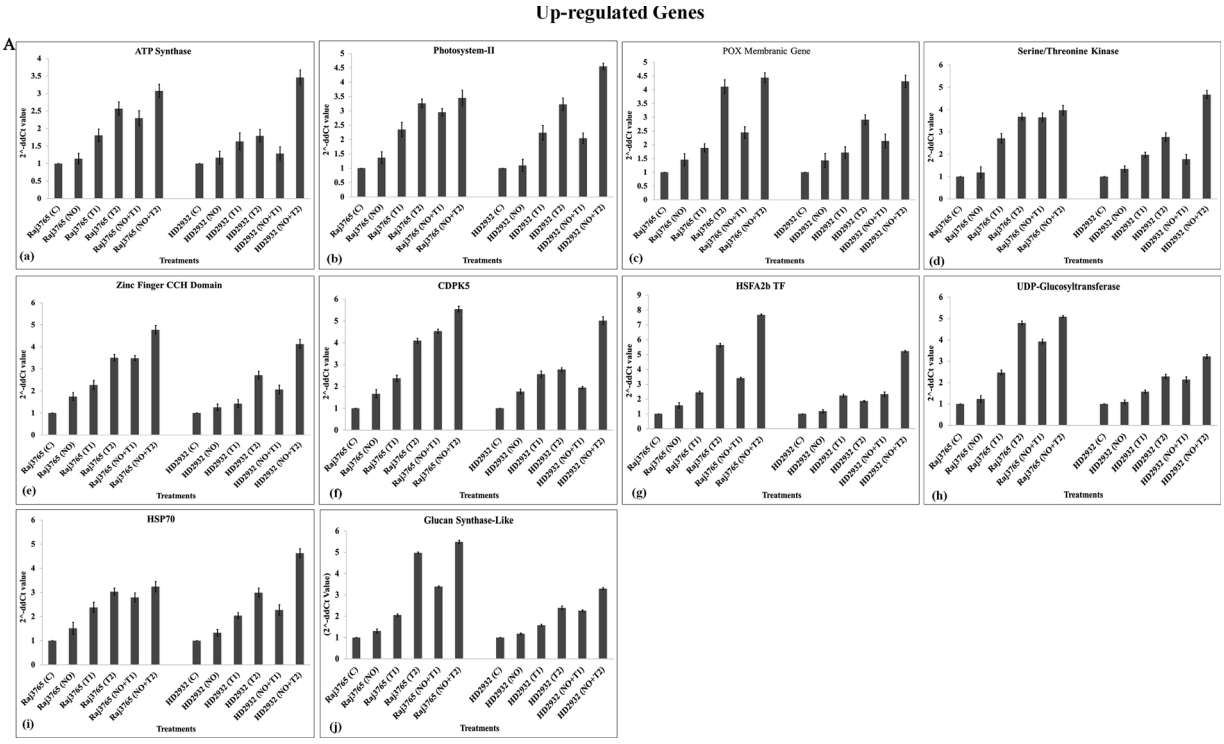

Fig. 2. (A). Expression analysis of upregulated stress-associated transcripts in contrasting wheat cultivars in response to NO and differential HS, Ten upregulated transcripts based on their digital fold expression were retrieved from whole transcriptome sequencing data of contrasting wheat cultivars under C and HS treatments executed in our lab; NO – 150 µM, C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h; β-actin gene (acc. no. AF282634) was used as endogenous control for normalizing the C_t value; Relative fold expression was calculated using the Pfaffl method (Pfaffl, 2001); Three biological replicates were used for the experiment, $P \leq 0.05$. (B). Expression analysis of downregulated stress-associated transcripts in contrasting wheat cultivars in response to NO and differential HS; Ten downregulated transcripts based on their digital fold expression were retrieved from whole transcriptome sequencing data of contrasting wheat cultivars under C and HS treatments executed in our lab; NO – 150 µM, C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h; β-actin gene (acc. no. AF282634) was used as endogenous control for normalizing the C_t value; The relative fold expression was calculated using the Pfaffl method (Pfaffl, 2001); Three biological replicates were used for the experiment, $P \leq 0.05$.

conductance and transpiration rate was observed higher in Raj3765, as compared to HD2932 in response to NO and differential HS (T_1 and T_2) during mealy-ripe stage. Uchida et al., (2002) reported that NO enhances the survival of green leaves tissues, and modulate the quantum yield for photosystem II as compared to non-treated controls, under salt and heat stresses in rice. Our findings are in conformity with the observation of Gong et al., (2017) who reported higher photosynthetic rate and chlorophyll content in NO-treated *Cucumis sativus* under Cd toxicity. Similar observation was reported in potato leaves infected with *Phytophthora infestans* (Lazalt et al., 1997). Therefore, we can conclude that NO helps to improve the photosynthetic rate by protecting the photosynthetic pigments in leaves of wheat crops.

3.2. Effect of NO and HS on expression of SAGs identified in wheat

We executed whole transcriptome sequencing of contrasting wheat cultivars under control and HS-treated conditions (BioProject acc. no. PRJNA171754). The data generated was annotated using different databases such as NCBI non-redundant, EnsemblPlant, etc. and further, identified ~1200 SAGs. We selected 10 upregulated transcripts (ATP synthase, PS-II, POX membrane, Serine threonine kinase (STK), Zinc Finger CCH Domain, CDPK5, HSFA2b, UDP Glucosyl transferase, HSP70 and Glucan Synthase-like) and 10 downregulated transcripts (Zinc Finger Protein Var3, Dnaj homolog, Myb Family TF, TF Myb44, Sucrose synthase2, sucrose phosphate synthase, sucrose transporter protien5, Delta Gliadin1, Alpha amylase inhibitor, alpha glucan water dikinase) based on their digital fold expression in response to differential HS (Table 1). We observed significant increase in the relative fold expression of ATP synthase gene under NO (150 μM) and differential HS (T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h) and also the maximum transcript was observed in response to NO + T_2 treatments in both cultivars. Similar pattern of expression of Photosystem-II, POX membrane and STK genes were observed in response to NO and differential HS (T_1 and T_2) in both cultivars [Fig. 2(A) b, c, d]. Similarly, zinc finger CCH domain and *CDPK5* showed maximum fold expression in response to NO + T_2 in Raj3765 and HD2932; Transcript abundance was observed more in Raj3875, as compared to HD2932 [Fig. 2(A) e & f)]. Similar pattern of expression of heat-responsive TF (*HSFA2b*) and UDP glucosyltransferase genes were observed in Raj3765 and HD2932 in response to NO and differential HS (T_1 and T_2). Though, NO was not much responsive in case of HD2932 under differential HS (T_1 and T_2) compared with Raj3765. Expression analysis of *HSP70* and Glucan synthase like genes showed significant variations in expression under NO and differential HS (T_1 and T_2) in both cultivars. We observed maximum relative fold expression of *HSP70* and *GSL* in response to NO + T_2 followed by T_2 only [Fig. 2(A) i & *j*)]. Grun et al. (2006) identified large number of NO-responsive genes in *Arabidopsis thaliana* which were involved in signal transduction, disease resistance and stress response, photosynthesis, cellular transport, and basic metabolism through a comprehensive transcript profiling by cDNA-amplification fragment length polymorphism (AFLP). NO has been reported to influence the activity of transcription factors and modulate the signaling cascades and expression of stress-associated genes (Bogdan, 2001). Parankusam et al., (2017) reported NO as one of the important signaling molecule involved in triggering the expression of various stress-associated genes in plants under HS.

Expression analysis of downregulated transcripts identified from *de novo* transcriptomic sequencing in wheat were analyzed for their expression variations in response to NO and differential HS (T_1 and T_2). NO was observed to modulate the expression of Zinc finger protein var3, Dnaj homolog and Myb family genes in both cultivars; maximum transcripts of all three genes were observed in HD2932, as compared to Raj3765. Under differential HS, we observed downregulation of Zinc finger protein, Dnaj Homolog and Myb family TF genes. The Zinc finger protein and Myb family genes showed minimum expression in response to NO + T_2 in Raj3765, whereas Dnaj homolog gene showed maximum downregulation in response to NO + T_1 in HD2932 [Fig. 2(B) a, b & c]. TF *Myb44* showed upregulation in response to NO in both cultivars, though the increase was higher in Raj3765 compared to HD2932. Under differential HS (T_1 and T_2), we observed downregulation of TF *Myb44* in both cultivars; maximum downregulation was observed in response to NO + T_2 in both cultivars. Similar pattern of expression of sugar transporter 5, delta gliadin1, and alpha amylase inhibitor genes were observed in both cultivars in response to NO and differential HS (T_1 and T_2). In case of sugar synthase 2, sucrose phosphate synthase, and alpha water glucan dikinase genes, an increase in the transcripts were observed in response to NO treatment; maximum relative fold expression was observed in HD2932, as compared to Raj3765. Further, we observed downregulation in all three genes under NO and differential HS (T_1 and T_2) treatments; maximum downregulation was observed in response to NO + T_2, except in case of sucrose phosphate synthase gene, where NO + T_1 causes maximum reduction in the transcript. NO suppresses the expression of genes associated with flowering, and thus regulate the photoperiodism in Arabidopsis (He et al., 2004).

3.3. Expression analysis of defense network of wheat cv. Raj3765 under NO and HS

We retrieved few SAGs cloned in our lab from wheat and sequenced using Sangers di-deoxy method. The SAGs selected for the NO (150 μM) and differential HS (T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h) analyses are - HSFs (*HSFA6e*, *HD97*), HSPs (*HSP90*, *HSP70*, *HSP26* and *HSP17*), antioxidant enzyme genes (*SOD*, *CAT* and *APX*) and signaling molecules/ catalytic chaperone (*MAPK* and *Rca*).

Expression analysis of HSFs showed significant increase in relative fold expression of both TFs (*HSFA6e* & *HD97*) in Raj3765; maximum transcript of *HD97* was observed in response to NO + T_2 treatment, as compared to *HSFA6e* TF (Fig. 3a). Similarly, expression analysis of HSPs (*HSP90*, *HSP70*, *HSP17* and *HSP26*) in Raj3765 showed increase in the relative fold expression in response to NO, as compared to control (Fig. 3b). High molecular weight HSPs (*HSP90* and *HSP70*) showed maximum fold expression in response to NO + T_2, whereas low molecular weight HSPs (*HSP26* and *HSP17*) showed maximum expression in response to NO + T_2 and NO + T_1, as compared to control. Expression analysis of antioxidant enzyme genes (*SOD*, *CAT* and *APX*) showed significant increase in the expression in Raj3765 under NO and differential HS (T_1 and T_2; Fig. 3c). Maximum fold expression of all three antioxidant enzyme genes was observed in response to NO + T_2, as compared to control. Ahmad et al., (2016) reported increase in transcripts of *SOD*, *CAT* and *APX* in NO-treated chickpea exposed to salt stress. The expression of Rca and MAPK genes were observed maximum in response to NO + T_2, as compared to control (Fig. 3d). An increase in the expression of SAGs was observed in contrasting wheat cultivars at different stages of growth and development under differential HS (Kumar et al., 2017).

3.4. Expression analysis of defense network of wheat cv. HD2932 under NO and HS

Expression analysis of *HSFA6e* and *HD97* showed increase in the relative fold expression in response to NO (150 μM) and differential HS (T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h); maximum transcripts were observed in response to NO + T_2, as compared to control. NO was observed to modulate the expression of *HSFA6e* and *HD97* TFs in wheat *cv*. HD2932 (Fig. 4a). Comparative analysis showed abundance of *HD97* transcript, as compared to *HSFA6e* under NO + T_2 treatment. Expression analysis of HSPs (*HSP90*, *HSP70*, *HSP26* and *HSP17*) showed very high fold increase in the expression of *HSP26* and *HSP17*, as compared to *HSP70* and *HSP90* under NO treatment (Fig. 4b). We observed increase in the expression of all four HSPs in response to T_1, T_2 and NO + T_1/T_2 treatments. *HSP90* and *HSP70* showed maximum relative fold

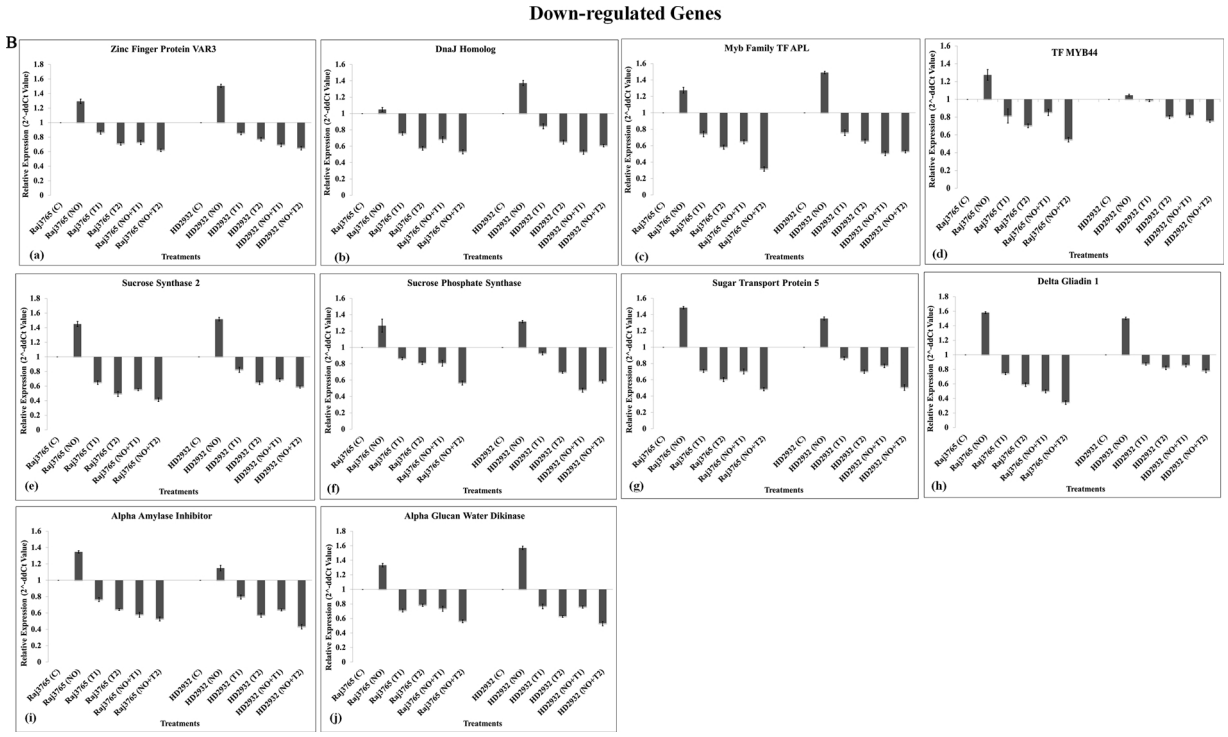

Fig. 2. (*continued*)

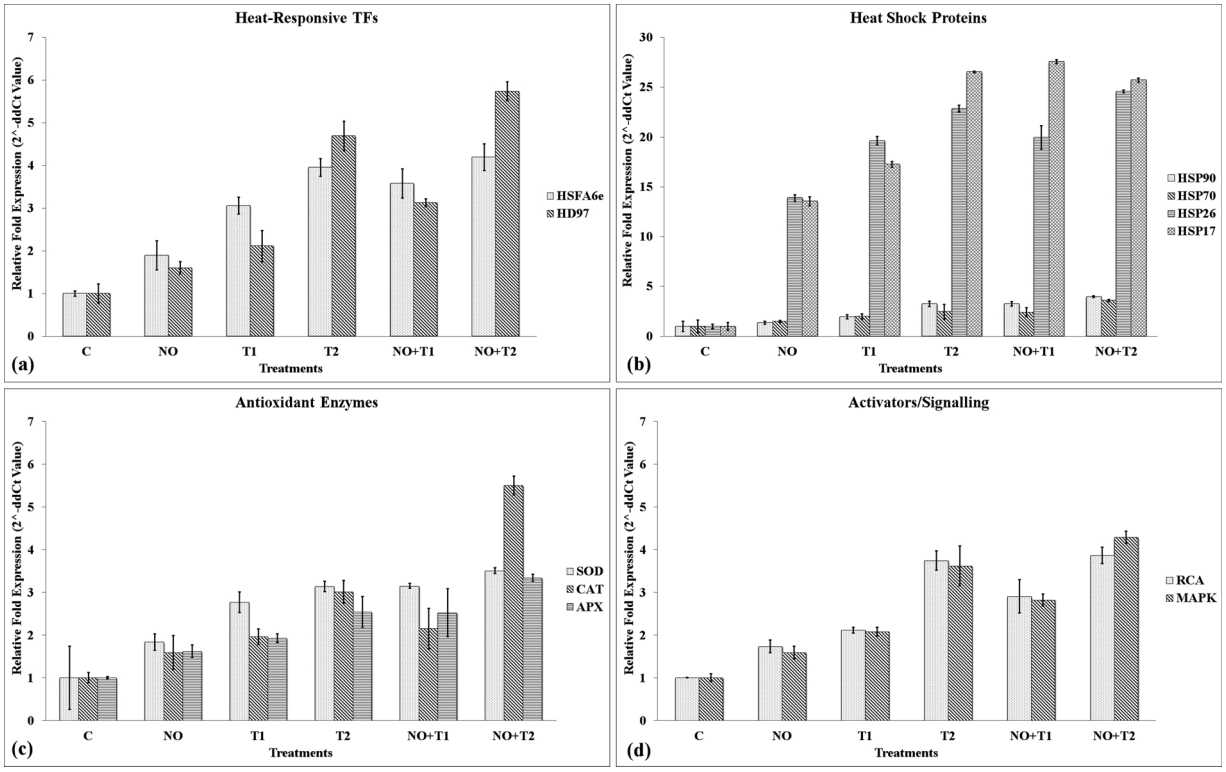

Fig. 3. Expression analysis of defense related stress-associated transcripts in wheat *cv.* Raj3655 in response to NO and differential HS, (a) expression of heat-responsive TFs (*HSFA6e* and *HD97*), (b) Heat Shock Proteins (*HSP90*, *HSP70*, *HSP26* and *HSP17*), (c) Antioxidant enzymes (*SOD*, *CAT* and *APX*), (d) Activators/signaling molecules (*Rca* and *MAPK*). Stress-associated transcripts were retrieved from whole transcriptome sequencing data of contrasting wheat cultivars under C and HS treatments executed in our lab; NO – 150 μM, C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h; β-actin gene (acc. no. AF282634) was used as endogenous control for normalizing the C_t value; The relative fold expression was calculated using the Pfaffl method (Pfaffl, 2001); Three biological replicates were used for the experiment, $P \leq 0.05$.

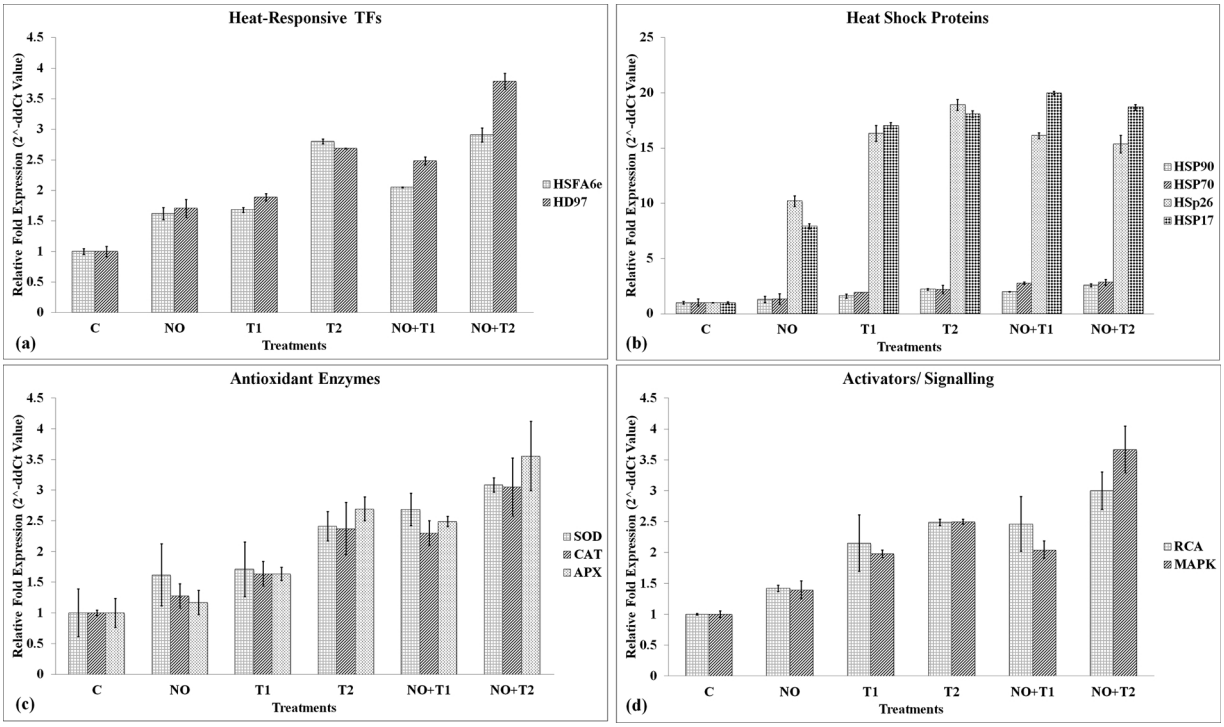

Fig. 4. Expression analysis of defense related stress-associated transcripts in wheat *cv*. HD2932 in response to NO and differential HS; (a) expression of heat-responsive TFs (*HSFA6e* and *HD97*), (b) Heat Shock Proteins (*HSP90*, *HSP70*, *HSP26* and *HSP17*), (c) Antioxidant enzymes (*SOD*, *CAT* and *APX*), (d) Activators/ signaling molecules (*Rca* and *MAPK*). Stress-associated transcripts were retrieved from whole transcriptome sequencing data of contrasting wheat cultivars under heat stress executed in our lab; NO – 150 μM, C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h; β-actin gene (acc. no. AF282634) was used as endogenous control for normalizing the C_t value; The relative fold expression was calculated using the Pfaffl method (Pfaffl, 2001); Three biological replicates were used for the experiment, $P \leq 0.05$.

expression in response to NO + T_2, whereas *HSP26* showed maximum relative fold expression under T_2, and *HSP17* under NO + T_1 treatments. Wang et al., (2014) reported that NO stimulate the DNA-binding activity of HSFs and the accumulation of HSPs in Arabidopsis seedlings through H_2O_2.

Expression analysis of antioxidant enzyme genes (*SOD*, *CAT* and *APX*) showed increase in the relative fold expression in response to NO treatment; *SOD* transcript was observed maximum compared with *CAT* and *APX* (Fig. 4c). The maximum relative fold expression of *SOD*, *CAT* and *APX* were observed in response to NO + T_2 treatments compared to control. Expression analysis of signaling molecule (*MAPK*) and catalytic chaperone (*Rca*) showed similar pattern of expression in response to NO and differential HS as explained above (Fig. 4d). Our findings are in conformity with the observation of Fancy et al., (2017). Even the NO-dependent oxidative burst in Tobacco was observed to be regulated by MAPK (Asai et al., 2008).

3.5. Effect of NO on antioxidant enzymes activity in contrasting wheat cultivars under HS

The leaf samples collected from wheat cultivars Raj3765 and HD2932 exposed to NO (150 μM) and differential HS (C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h) during pollination, milky-ripe and mealy-ripe stages of growth were analyzed for activity assay of SOD and CAT – two prominent enzymes involved in production and neutralization of hydrogen peroxide generated under differential HS – a biochemical marker for assessing the tolerance status of the plant.

We observed significant variations ($p \leq 0.05$) in the activity of SOD in Raj3765 and HD2932 in response to NO and differential HS treatments; maximum SOD activity was observed in response to T_2 in Raj3765 (11.2 U/mg protein) and T_1 in HD2932 (7.99 U/mg protein) during pollination stage (Fig. 5a). During milky-ripe sub-stage of grain-filling, Raj3765 showed maximum SOD activity in response to T_1 (12.0 U/mg protein) and HD2932 under T_2 treatments (10.8 U/mg protein; Fig. 5b). SOD activity was observed maximum in response to T_2 in both the cultivars (14.0 and 13.1 U/mg protein) during mealy-ripe stage (Fig. 5c). NO was observed to modulate the activity of SOD in both the cultivars; percent increase was observed maximum during milky-ripe stage. NO accumulation has been reported to induce the expression of genes associated with defense proteins during stress episodes and recovery (Fancy et al., 2017). NO treatment has been reported to increase the activities of superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase and catalase in Arabidopsis (Xuan et al., 2010), wheat (Karpets et al., 2012), maize (Mika and Lüthje, 2003) and chrysanthemum (Yang et al., 2011).

Catalase assay showed marked significant variations ($p \leq 0.05$) in the activity in response to NO and differential HS (T_1 and T_2) in contrasting wheat cultivars at different stages of growth and development. CAT activity was observed maximum in response to T_2 in Raj3765 (15.5 U/mg protein) and HD2932 (11.8 U/mg protein) (Fig. 5d). Similar pattern of CAT activity was observed in Raj3765 and HD2932 during milky-ripe and mealy-ripe sub-stages of grain-filling in response to NO and differential HS (T_1 and T_2) (Fig. 5e & f). NO was observed to modulate the CAT activity in both cultivars, however Raj3765 was observed more responsive compared to HD2932. NO has been reported to increase the expression and activities of antioxidant enzymes in wheat seedling exposed to Al toxicity (Sun et al., 2018).

3.6. Effect of NO on GPX and total antioxidant potential (TAP) of wheat under HS

The leaves collected from wheat cultivars Raj3765 and HD2932 exposed to NO and differential HS (C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h) during pollination, milky-ripe and mealy-ripe stages were

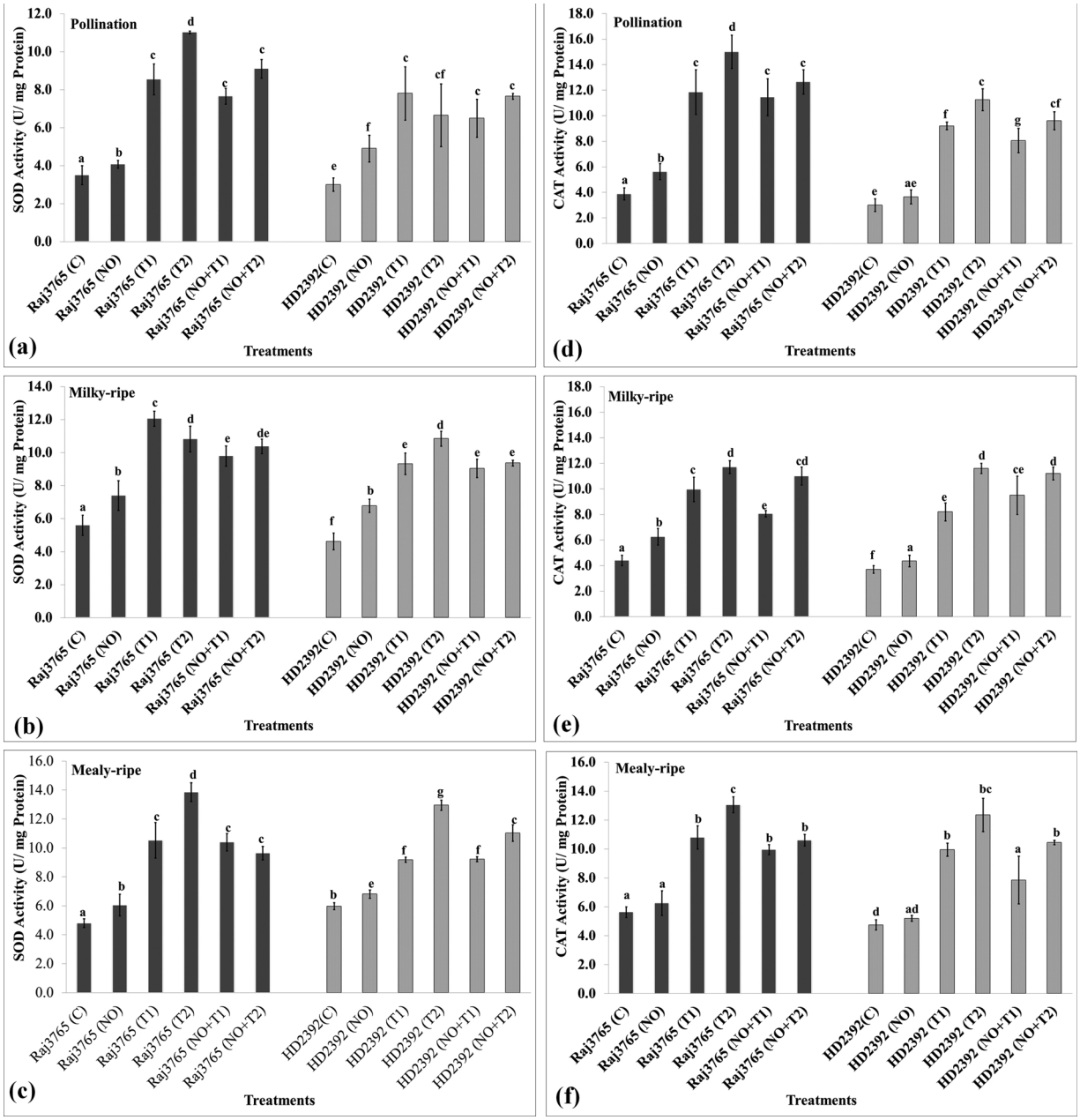

Fig. 5. Effect of NO on activities of superoxide dismutase and catalase in contrasting wheat cultivars at different stages under differential HS; (a) SOD activity in Raj3765 and HD2932 during pollination stage, (b) SOD activity in Raj3765 and HD2932 during milky-ripe stage, (c) SOD activity in Raj3765 and HD2932 during mealy-ripe stage, (d) CAT activity in Raj3765 and HD2932 during pollination stage, (e) CAT activity in Raj3765 and HD2932 during milky-ripe stage, (f) CAT activity in Raj3765 and HD2932 during mealy-ripe stage; NO – 150 µM, C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h; Three biological replicates were used for the estimation, Variables above histogram shows SEM (n = 3), significance level at $P \leq 0.05$.

analyzed for the GPX activity and total antioxidant potential – indirect markers of thermotolerance of wheat.

During pollination stage, GPX assay showed maximum activities in response to T_2 in Raj3765 (152.5 U/mg protein) and NO + T_2 in HD2932 (135.2 U/mg protein; Fig. 6a). GPX assay during milky-ripe and mealy-ripe stages showed maximum GPX activities in response to T_2 in both the cultivars; the variations were observed significant in response to treatments ($p \leq 0.05$) (Fig. 6b & c). GPX was observed very responsive to NO treatment in both cultivars at different stages of growth and development. Shi et al., (2005) reported increase in the activities of SOD, catalase, and peroxidase in beans exposed to NO under UV-B treatment which is in conformity with the observation in present investigation.

TAP assay during pollination stage showed maximum scavenging potential in response to T_2 in Raj3765 (24.5 mM/g fresh weight) and HD2932 (23.9 mM/g fresh weight; Fig. 6d). Similarly, TAP assay during milky-ripe and mealy-ripe stages showed maximum scavenging potential in response to T_2 in both cultivars (Fig. 6e & f). Raj3765 showed high TAP, as compared to HD2932. The variations in the TAP at different stages of growth were observed significant ($p \leq 0.05$) in both cultivars. Exogenous application of NO was observed to decrease the scavenging potential (TAP) of both cultivars at different stages of growth and development. The similar effect was observed in response to combined treatments of NO and differential HS (T_1 and T_2) in both cultivars. The findings is in conformity with the observation of Hasanuzzaman et al., (2013) who reported increase in the antioxidants especially ascorbate and glutathione contents in response to NO treatment in wheat seedling under HS.

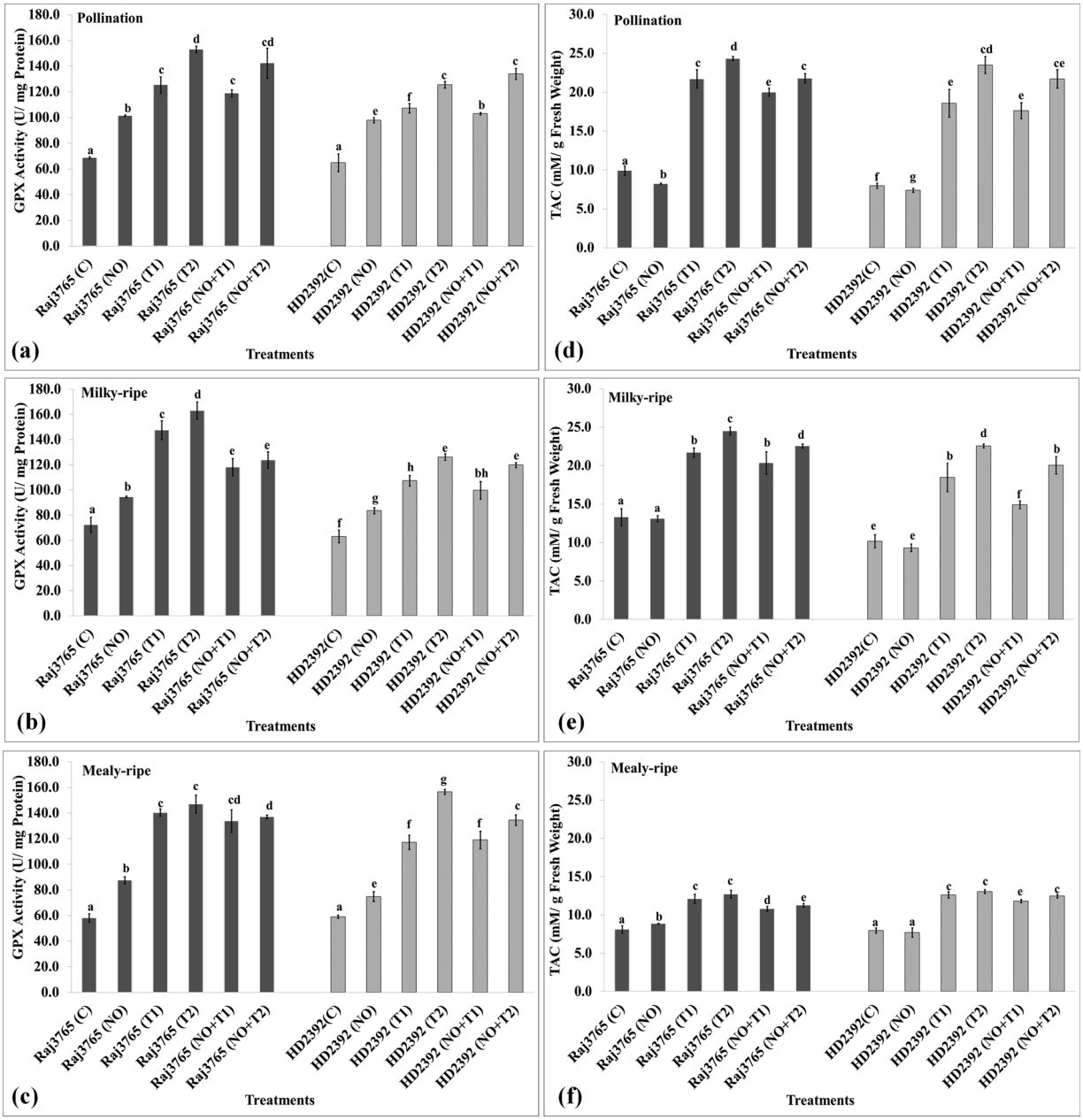

Fig. 6. Effect of NO on activities of guaiacol peroxidase and total antioxidant potential in contrasting wheat cultivars at different stages under differential HS; (a) GPX activity in Raj3765 and HD2932 during pollination stage, (b) GPX activity in Raj3765 and HD2932 during milky-ripe stage, (c) GPX activity in Raj3765 and HD2932 during mealy-ripe stage, (d) TAC in Raj3765 and HD2932 during pollination stage, (e) TAC in Raj3765 and HD2932 during milky-ripe stage, (f) TAC in Raj3765 and HD2932 during mealy-ripe stage; NO – 150 µM, C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h; Three biological replicates were used for the estimation, Variables above histogram shows SEM (n = 3), significance level at $P \leq 0.05$.

3.7. Effect of NO on accumulation of proline and free amino acid in wheat under HS

The leaves collected from wheat cultivars Raj3765 and HD2932 exposed to NO and differential HS (C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h) during pollination, milky-ripe and mealy-ripe stages were analyzed for the proline and free amino acid accumulation - a prominent osmolyte and pathway modulator – in wheat.

Proline profiling during pollination stage showed significant variations ($p \leq 0.05$) in both cultivars in response to treatments. Maximum proline accumulation was observed in response to T_2 in Raj3765 (1.15 µM/ g fresh weight) and HD2932 (1.05 µM/ g fresh weight; Fig. 7a). Similarly, during milky-ripe and mealy-ripe stages, we observed maximum accumulation of proline in response to T_2 treatments (Fig. 7b & c). The variations were observed significant ($p \leq 0.05$) in response to treatments. NO was observed to modulate the accumulation of proline at different stages of growth and development which is in conformity with the findings of Alamri et al., (2018) who reported increases in the accumulation of proline and thermotolerance of *Vicia faba* pre-treated with NO under stress (Alamri et al., 2018).

Free amino acid estimation during pollination showed maximum accumulation in response to T_2 in Raj3765 (4.4 mg/ g fresh weight) and HD2932 (3.2 mg/ g fresh weight) (Fig. 7d; though the variations were observed non-significant ($p \leq 0.05$). During milky-ripe stage, we observed maximum accumulation of free amino acid in response to NO + T_2 in Raj3765 (4.5 mg/ g fresh weight) and under T_2 in HD2932 (3.2 mg/ g fresh weight; Fig. 7e). Similar pattern of free amino acid accumulation were observed in both cultivars during mealy-ripe stage;

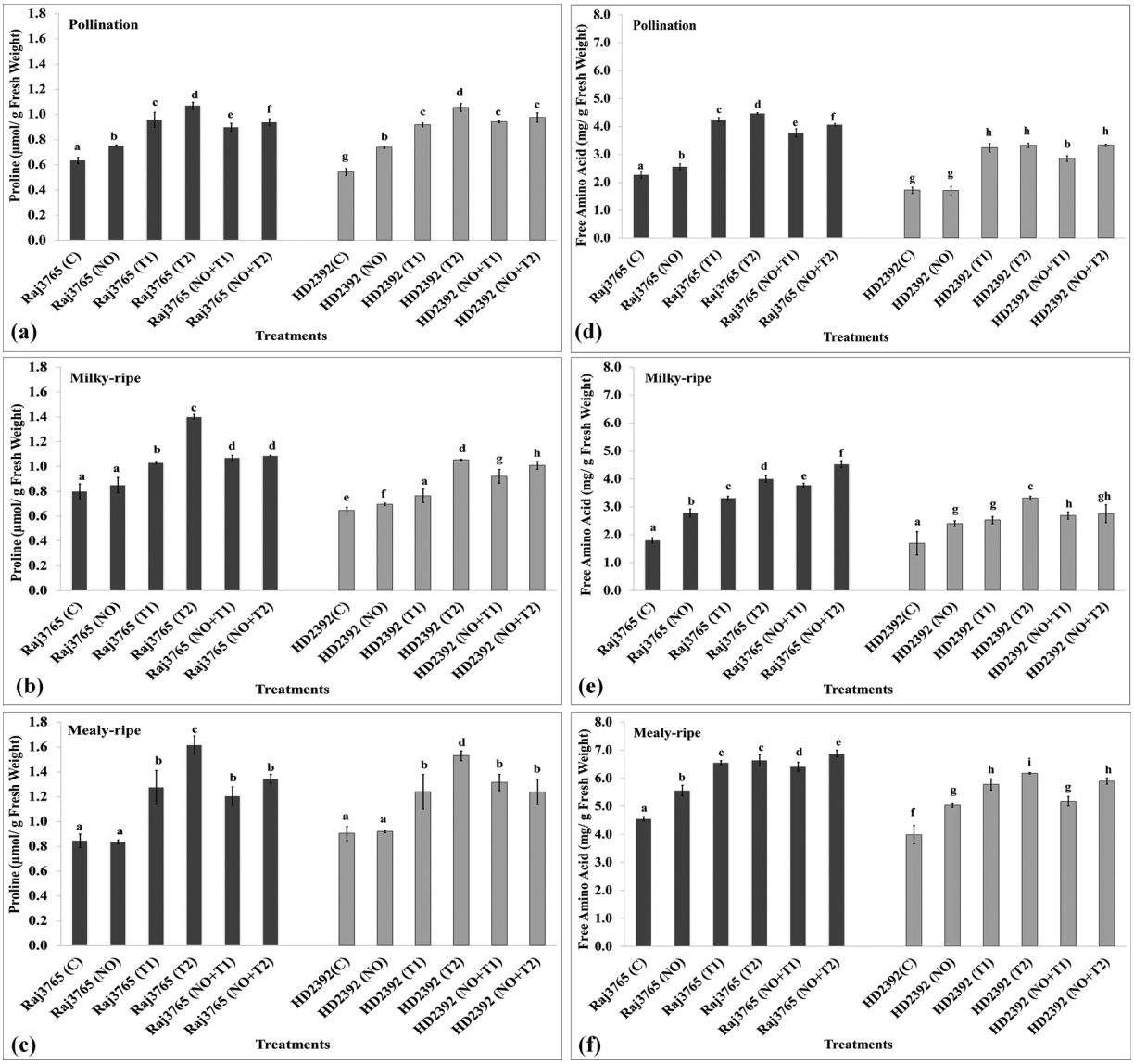

Fig. 7. Effect of NO on accumulation of proline and free amino acid in contrasting wheat cultivars under differential HS, (a) Proline accumulation in Raj3765 and HD2932 during pollination stage, (b) Proline accumulation in Raj3765 and HD2932 during milky-ripe stage, (c) Proline accumulation in Raj3765 and HD2932 during mealy-ripe stage, (d) Free amino acid accumulation in Raj3765 and HD2932 during pollination stage, (e) Free amino acid accumulation in Raj3765 and HD2932 during milky-ripe stage, (f) Free amino acid accumulation in Raj3765 and HD2932 during mealy-ripe stage; NO – 150 µM, C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h; Three biological replicates were used for the estimation, Vertical bars indicate *s.e* (n = 3), significance level at $P \leq 0.05$.

the variations were observed non-significant ($p \leq 0.05$) (Fig. 7f). Free amino acid was observed to increase in different tissues of wheat under differential HS (Kumar et al., 2017).

3.8. Effect of NO and HS on quality of developing grains in contrasting wheat cultivars

We collected the developing grains from wheat cultivars Raj3765 and HD2932 treated with NO followed by differential HS (T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h). The purified prolamins fraction of proteins were isolated and used for immunoblot assay using polyclonal anti-gliadin specific antibody (Sigma-Aldrich, UK). Immunoblot assay of samples collected from Raj3765 and HD2932 during milky-ripe stage showed very prominent and intact bands of gliadin protein (~47 kDa) on the SDS-PAGE gel. No significant variations were observed in intensity of gliadin protein bands in case of Raj3765, whereas HD2932 showed increase in the intensity of gliadin protein with temperature. We observed maximum accumulation of gliadin protein in response to NO + T_2 in HD2932 (Fig. 8a). During mealy-ripe stage, we observed significant increase in the accumulation of gliadin protein with temperature in both cultivars; maximum gliadin protein accumulation was observed in response to NO + T_2 treatments in both cultivars (Fig. 8b). NO was observed to modulate the accumulation of prolamins fraction of protein in developing wheat grains under differential HS. The gliadin protein accumulation was observed more in developing grains of Raj3765, as compared to HD2932 under differential HS. Similar observations in wheat were reported by Zhang et al., (2018).

3.9. Effect of NO and differential HS on amylolytic activity in developing grains of wheat

The grains collected from wheat cultivars Raj3765 and HD2932 treated with NO (150 µM) and differential HS (T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h) during the seed hardening sub-stage of grain-filling was used for extraction of alcohol soluble prolamins fraction of protein. The isolated prolamins were analyzed for *in-gel* activity of amylases. We observed

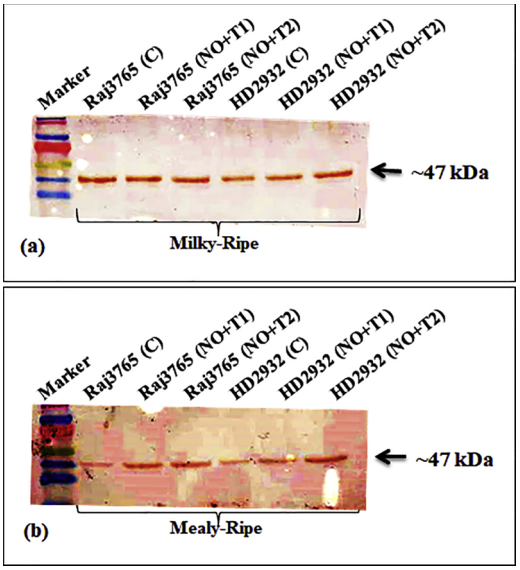

Fig. 8. Immunoblot assay for characterizing the effect of NO and differential HS on accumulation of gliadin protein in developing wheat grains; Samples were collected during (a) milky-ripe, and (b) mealy-ripe sub-stages of grain-filling; Polyclonal anti-gliadin antibody (Sigma-Aldrich, UK) was used as primary antibody and HRP conjugated secondary antibody was used for the screening.

two prominent bands in response to NO and differential HS (T_1 and T_2) treatments in both cultivars (Fig. 9a & b). The intensity of protein band in each lane was observed low in Raj3765, as compared to HD2932 under NO and differential HS (T_1 and T_2) (Fig. 9c & d). This makes us to conclude that Raj3765 has low amylolytic activity compared with HD2932. NO was observed to decrease the amylolytic activity, whereas differential HS was observed to increase the amylase activity in both cultivars. The observation was in conformity with the findings of Hu et al., (2007) who reported the increase in the amylolytic activity in wheat seeds when exposed to NO.

3.10. In vitro activities of α/ β-amylases in developing wheat grains under NO and HS

We collected the prolamins fraction of developing grains of wheat cultivars Raj3765 and HD2932 exposed to NO (150 μM) and differential HS (C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h). Total amylolytic activity in developing grains of wheat *cv*. Raj3765 was observed maximum in NO + T_1 treatment (1.15 U/mg proteins; Fig. 10a). Similar pattern of activity of α-amylase was observed in the developing grains of wheat *cv*. Raj3765 under NO and differential HS treatments. Maximum α-amylase activity was observed in response to NO + T_1 (0.86 U/mg proteins). The variation in the activity of α-amylase was observed significantly ($p \leq 0.05$) in response to treatments. β-amylase showed maximum activity in response to T_1 (0.36 U/mg proteins). β-amylase showed significant variation in the activity in response to NO and differential HS. Deng and Song, (2012) observed the increase in seed germination and activity of β-amylase in *Lactuca sativa* seeds imbibed with sodium nitroprusside.

Total amylolytic activity in developing grains of wheat *cv*. HD2932 showed maximum activity in response to NO (1.25 U/mg proteins; Fig. 10b). We observed decrease in the activity of α-amylase in developing grains of HD2932 in response to NO and differential HS, as compared to control. β-amylase showed maximum activity in response to T_1 (0.31 U/mg proteins). We observed significant variations ($p \leq 0.05$) in the amylolytic, α/β - amylases activities in developing grains of wheat *cv*. HD2932 under NO and differential HS (T_1 and T_2). Maurice et al. (2016) reported the increase in α/β - amylases activities in alfalfa seedling under cadmium stress.

Starch was estimated in the developing grains of wheat cultivars Raj3765 and HD2932 exposed to NO and differential HS (T_1 and T_2). In case of Raj3765, maximum starch was observed in response to NO + T_2 (75.2%) treatment (Fig. 10c). HD2932 showed maximum starch content in response to NO (78%). The variations in the starch content in the developing grains of both the cultivars were observed highly significant ($p \leq 0.05$). The accumulation of starch was observed significantly higher in both the cultivars. The result was in conformity with the observation of Maurice et al. (2016) in alfalfa seedling.

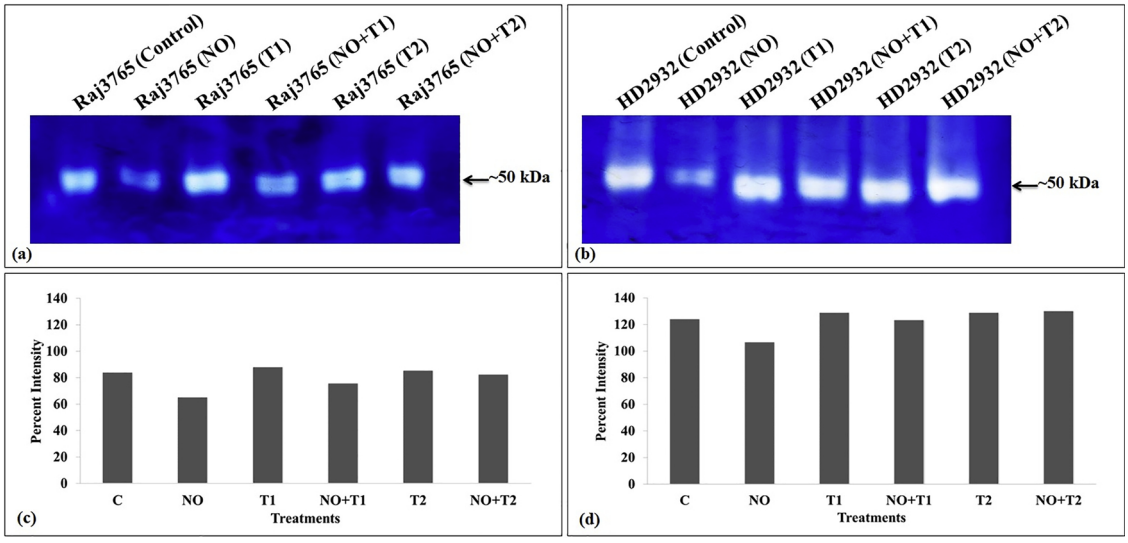

Fig. 9. *In-gel* activity assay of amylases in the developing grains of contrasting wheat cultivars in response to NO (150 μM) and differential HS (T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h); (a) Electrophoretic mobility profiling of amylases in grains of wheat *cv*. Raj3765 under NO and differential HS, (b) Electrophoretic mobility profiling of amylases in grains of wheat *cv*. HD2932 under NO and differential HS, (c & d) Densito-analysis of intensity of amylases band visualized on the gel and expressed in terms of percent intensity.

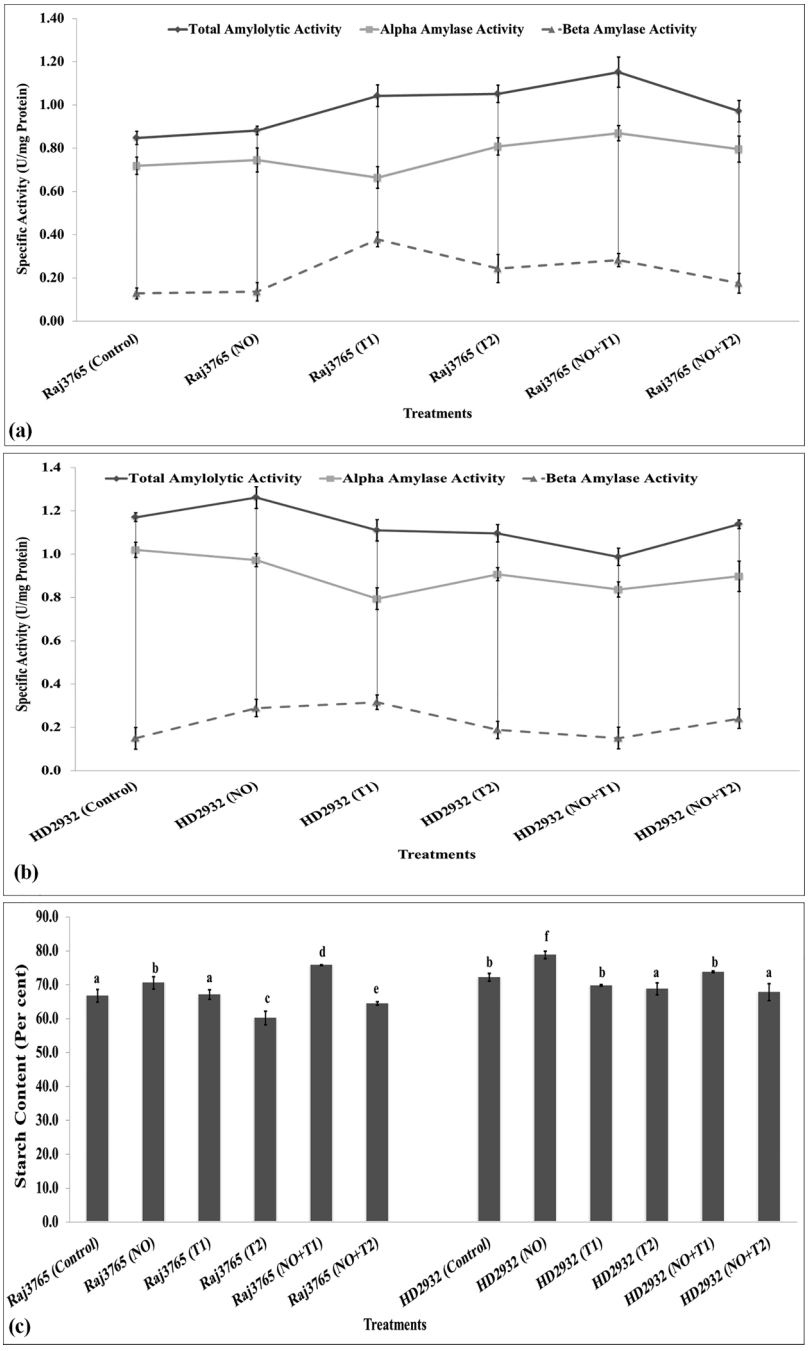

Fig. 10. Effect of NO and differential heat stress on amylolytic activity in the developing grains of contrasting wheat cultivars, (a) Amylolytic activity in the grains of wheat *cv*. Raj3765 in response to NO and differential HS; (b) Amylolytic activity in the grains of wheat *cv*. HD2932 under NO and differential HS; NO - 150 μM, DHS (C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h); Three biological replicates were used for the analysis; Hardened seeds were used for the activity assay, Vertical bars indicate *s.e* (n = 3), significance level at $P \leq 0.05$.

3.11. Effect of NO and HS on soluble protein accumulation in the wheat grains

The soluble protein extracted from the grains of wheat cultivars Raj3765 and HD2932 exposed to NO (150 μM) and differential HS (C - 22 ± 2 °C, T_1 - 30 °C, 1 h, T_2 - 38 °C, 1 h) were estimated. We observed slight increase in the protein content in the seeds of wheat *cv*. Raj3765 in response to NO and T_1 (16.2 and 16.1 mg/g flour) (Fig. 11). In case of HD2932, we observed decreased protein content in the seeds treated with NO and differential HS (T_1 and T_2). The variations were observed non-significant in both the cases ($p \leq 0.05$). Zhang et al., (2018) reported increase in the contents of gluten, gliadin, globulin, and albumin proteins by high-temperature stress in wheat kernel. NO, being the signaling molecule, has demonstrated its potential role in terms of triggering various physiological and biochemical processes associated with thermotolerance inside the plant system having beneficial effect on the end produce.

3.12. Model elucidating the role of NO in defense and quality of developing wheat grains

The present investigation was executed to understand the effect of exogenous NO on defense network and quality of wheat grains under differential HS at different stages of growth and development

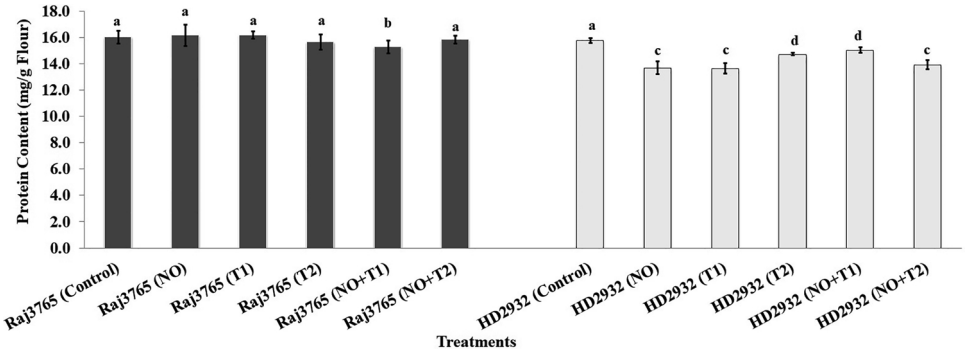

Fig. 11. Effect of NO and differential heat stress on soluble protein content in grains of contrasting wheat cultivars; Fully developed grains of Raj3765 and HD2932 were used for the estimation; Three biological replicates were used for the experiment; Vertical bars indicate *s.e* (n = 3), significance level at P ≤ 0.05.

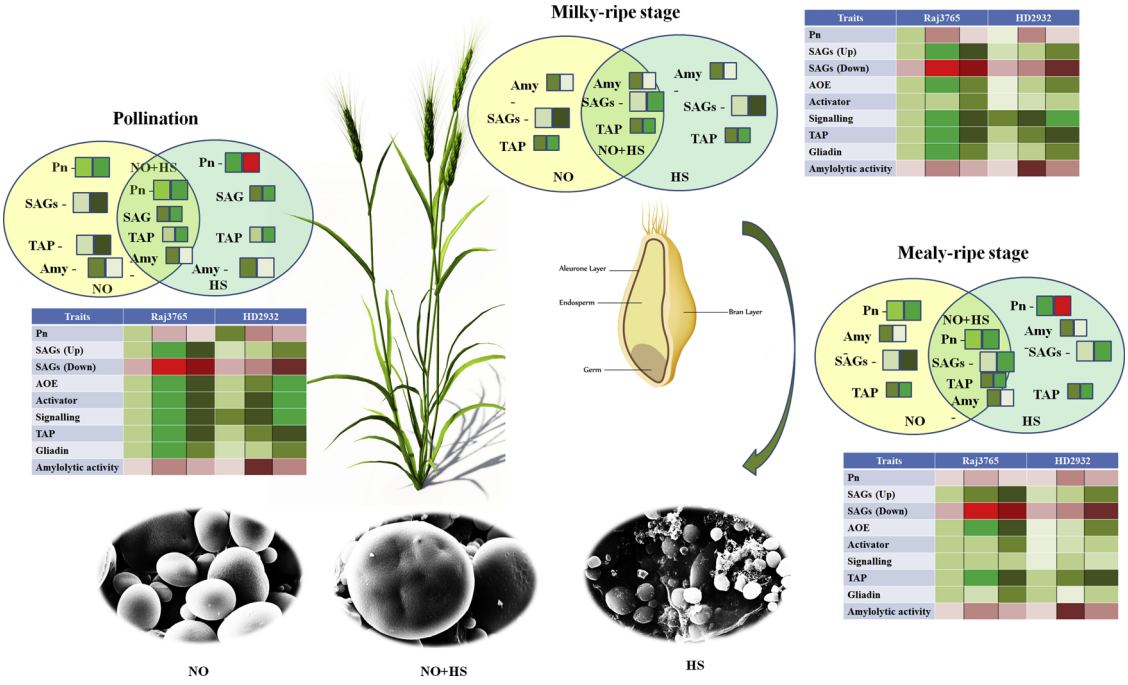

Fig. 12. Model depicting the effect of exogenous NO on defense network and grain quality related parameters under differential HS; NO – 150 µmol, HS - 30˚/38 ˚C, 1 h, Two wheat cultivars Raj3765 (thermotolerant) and HD2932 (thermosusceptible) were used for the analysis, Color gradient depict the relative fold expression, Green – up-regulation, Red – down-regulation, P_n – photosynthetic rate, Amy – amylases activity, TAP – total antioxidant potential SAGs – stress associated genes, AOE – antioxidant enzymes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Pollination, milky-ripe and mealy-ripe stages; Fig. 12). During pollination stage, we observed increase in the expression of SAGs like TFs, HSPs, antioxidant enzyme genes, signaling molecules and activator genes under NO treatment, in order to mitigate the effect of HS; the expression was higher in Raj3765, as compared to HD2932. Combined treatment of NO + HS in wheat was observed more effective in mitigating the effect of terminal HS in both cultivars. Similar pattern of expression and activities of SAGs and SAPs were observed during milky-ripe stage of grain development. The quality of grains was observed improved in NO treated wheat in terms of gliadin protein, soluble protein and accumulation of high quality starch. The total amylolytic activity was observed decreased in developing grains under NO treatment, contrary to the effect of HS. During mealy-ripe stage, NO was not much effective in maintiang the Pn, expression and activities of SAGs and SAPs against HS. Though, the quality of grain was better in terms of proteins and starch during mealy-ripe stage. Even the amylases activity was observed lower in response to NO under HS, as evident from the synthesis of high quality starch granules (Fig. 12). The effect of NO was observed more pronounced in Raj3765 *cv.*, as compared to HD2932 under HS.

4. Conclusion

NO plays pivotal role in maintaining the tolerance of cells under HS by triggering various biochemical and molecular mechanisms in wheat crops. In the present study, we observed very high relative fold expression of stress-associated genes in response to NO (150 µM) and differential HS (T_1 - 30 ˚C, 1 h, T_2 - 38 ˚C, 1 h) in contrasting wheat cultivars; sHSPs showed maximum transcripts accumulation, compared with other SAGs. The expression of heat-responsive TFs, HSPs and pathways associated genes were observed triggered by NO in wheat under differential HS. The activities of SOD, CAT, and GPX were observed higher in NO treated wheat followed by accumulation of proline and free amino acid under differential HS. We observed increase in accumulation of gliadin protein in wheat endospermic tissue under NO + T_2 in both the thermotolerant (Raj3765) and thermosusceptible (HD2932) cultivars. The activities of α/β-amylases were observed repressed in NO treated wheat causing balanced accumulation of starch and soluble proteins in seeds under differential HS. The beneficial effect of NO was observed more pronounced in thermotolerant *cv.* (Raj3765), as compared to thermosusceptible (HD2932). Exogenous application of

NO is a cost-effective technology to mitigate the problem of terminal HS in wheat maintaining the quality of the grains.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2018.11.016.

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